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Original article

Contextual regulation of pancreatic cancer stem cell phenotype and radioresistance by pancreatic stellate cells

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ABSTRACT

Background and purpose: Progression of pancreatic ductal adenocarcinoma (PDAC) is promoted by desmoplasia induced by pancreatic stellate cells (PSC). Contributory to this progression is epithelial mesenchymal transition (EMT), which shares many characteristics with the cancer stem cell (CSC) hypothesis. We investigated the role of these processes on the radioresponse and tumorigenicity of pancreatic cancer cells.

Materials and methods: We used an in vitro sphere model and in vivo xenograft model to examine the role of PSC in EMT and CSC processes.

Results: We demonstrated that PSC enhanced the CSC phenotype and radioresistance of pancreatic cancer cells. Furthermore, the expression of several EMT and CSC markers supported enhanced processes in our models and that translated into remarkable in vivo tumorigenicity. Multi-dose TGFβ neutralizing antibody inhibited the EMT and CSC processes, sensitized cells to radiation and reduced in vivo tumorigenicity. A proteomic screen identified multiple novel factors that were regulated by PSC in pancreatic cells.

Conclusion: These results are critical in highlighting the role of PSC in tumor progression and radioresistance by manipulating the EMT and CSC processes. TGFβ and the novel factors identified are important targets for better therapeutic outcome in response to PSC mediated mechanisms.

Pancreatic ductal adenocarcinoma is an aggressive and highly lethal disease, with less than 1% 5-year survival. The poor prognosis of PDAC is attributed in part to the presence of a strong desmoplastic reaction driven by pancreatic stellate cells (PSC), which are the main source of excessive extracellular matrix production in chronic pancreatitis and pancreatic adenocarcinoma [3]. The presence of PSC is advantageous for tumor growth in vivo [14].

A subpopulation of cells called cancer stem cells (CSC) has been isolated from PDAC [15]. CSCs are thought to be a determining factor in chemo and radioresistance [7]. However, the model used is important in determining the outcome [1]. A link between CSC and epithelial mesenchymal transition (EMT) has been demonstrated [19]. Inducers of EMT include transforming growth factor β (TGFβ) [9,26], which is also involved in PSC activation [18]. Activated PSC drive the desmoplastic reaction characteristic of pancreatic cancer resulting in extensive collagen types I, III and fibronectin production [24]. Furthermore, in vivo models of pancreatic cancer show an EMT defect that is attributed to deletion in TGFβ signaling including DPC4/smad4 and transcriptional intermediary factor (TIF)-1γ [31].

In this study, we investigated the role of PSC in the induction of CSC and EMT and the effect on the radioresponse of pancreatic cancer cells in vitro and in vivo. An improved understanding of the role of stellate cells in the contextual induction of stem cell phenotype is critical in the development of more effective treatments.

Materials and methods

Complete description of Materials and methods is provided in the Supplementary Materials due to space limitation.

Cell culture

The human pancreatic cancer cell lines PSN-1, Panc-1 and MiaPaCa-2 were originally obtained from the American Type Tissue Collection. The human pancreatic stellate cell line (hPSC) was kindly provided by Dr. Atsushi Masamune [20]. The LTC-14 cells

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used in the supplementary figures were rat stellate cells [30]. The different cell lines were cultured in the recommended medium as suggested by the supplier.

Indirect co-culture of pancreatic cancer cells-pancreatic stellate cells and sphere formation

Human PSC were seeded in chemically-defined medium and single pancreatic cancer cell suspensions were resuspended in a mixture of Matrigel (BD Biosciences) and serum free medium, and plated into culture inserts of 1.0 μm pore size (BD Biosciences). After 10 days of incubation, spheres were counted and measured for size. Spheres were then retrieved using Matrigel Recovery Solution (BD Biosciences) according to the manufacturer's instructions.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting

Spheres protein extracts were resolved on a NuPAGE 4–12% Bis–Tris mini gel (Invitrogen) and transferred onto a Hybond-C Extra membrane (Amersham BioSciences). Membranes were cut in several sections and incubated with several antibodies for quantitative analysis of the different samples on the same membrane using chemiluminescence (ECL, Pierce). Supplementary Table 1 summarizes all antibody concentrations used for the corresponding technique. ImageJ was used to evaluate the densitometric differences employing integrated intensity.

Immunohistochemical and immunofluorescence staining of formalin-fixed frozen tissues

All animal procedures were carried out in accordance with current U.K. legislation under an approved project license. Excised tumors were fixed in a 4% neutral solution of formaldehyde and cut into 10 μm sections. The antibodies used and their working concentrations are described in Supplementary Table 1. Sections used for immunohistochemistry were counterstained with hematoxylin and viewed using a standard microscope. Sections used for immunofluorescence were viewed under either an epifluorescent or confocal microscope. ImageJ was used to evaluate the average pixel density differences in whole images.

Flow cytometry

Panc-1 spheres grown with or without human PSC were mechanically dissociated and washed twice with ice-cold phosphate-buffered saline (PBS). Then they were incubated with the non-enzymatic Cell Recovery solution (BD Biosciences) for up to 1 h at 4 °C on a rotary shaker. Cells obtained from mechanically dissociated spheres were stained with the corresponding antibodies, as outlined in the Supplementary Table 1, and analyzed using BD FACSCalibur machine.

Enzyme-linked immunosorbent assay (ELISA) and neutralizing TGFβ antibody treatment

Quantikine Human TGFβ1 Immunoassay (R&D Systems, Inc.) was performed as described by the manufacturer. E-Cadherin ELISA was assayed as described by the manufacturer (MesoScale).

In vivo tumorigenicity and TCD50 assay

All animal procedures were carried out in accordance with the current UK legislation under an approved project license. For the in vivo tumorigenicity assay, spheres were disaggregated, injected and followed up to 6 months. For the TCD50 experiments, single

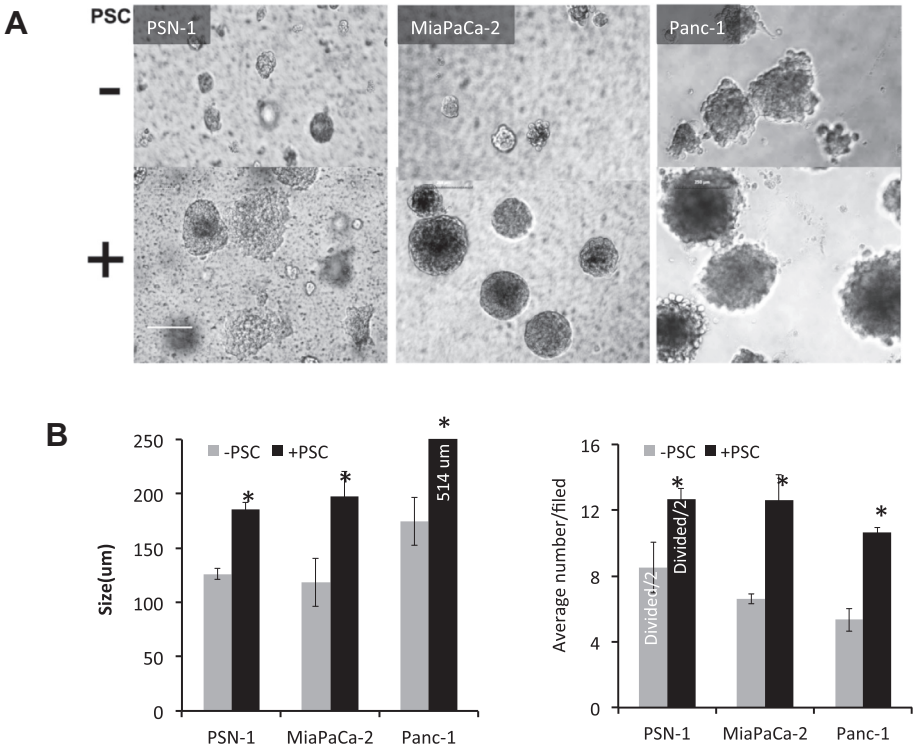

Fig. 1. The effect of human pancreatic stellate cells (PSC) on the sphere forming ability of three pancreatic cancer cell lines. Panel A shows representative pictures of spheres established from pancreatic cancer cell lines co-cultured with or without human PSC, 7–12 days after plating into Matrigel. Panel B is a numerical representation for the pictures in panel A as assessed for both size and number. The asterisks represent statistical significance for differences using the Student's *t*-test ($\alpha = 0.05$). The error bars represent SE of different wells from three different plates.

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